The Functional Role of miR-155 in Natural Killer Cells

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by
Srirama Josyula

The Ohio State University
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Project Advisor: Michael A. Caligiuri, M.D., Department of Molecular Virology, Immunology and Medical Genetics

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Abstract:

MicroRNAs (miRs) are small, non-coding RNA molecules with important regulatory functions. It appears that miRs are involved in lymphocyte, specifically Natural Killer (NK) cell, homeostasis, activation, and over-activation leading to malignancy. The human NK cell is controlled by a milieu of activating and inhibitory signaling that explain its highly regulated role in the immune response. The role of miR-155 in NK cells is complex and has direct effects on a variety of signaling pathways. In terms of external control on miR-155, we note that miR-155 is synergistically induced in primary human NK cells after co-stimulation with IL-12 and IL-18 cytokines. Additionally, miR-155 directly targets SHIP1, a molecule that is an important negative regulator of pathways involving survival and proliferation. **Based on this preliminary data, we hypothesize that miR-155 plays a key role in regulation of activating pathways in NK cells.**

In order to study the role of miR-155 in NK cells, a miR-155 transgene (tg) overexpression was driven via the *lck* promoter in C57BL/6 mice. Once again, MiR-155 targets and downregulates the inositol phosphatase SHIP1, which is a negative regulator of NK cell activation. Phenotypically, miR-155 tg mice have enlarged spleens with greater absolute number and percentage of NK cells. Using purified NK cells from spleens of miR-155 Tg and wild type (wt) mice, we observed that miR-155 upregulation increases NK cell cytotoxic activity against tumor cells utilizing both spontaneous cytotoxic and antibody dependent cell mediated cytotoxic pathways. NK cells from miR-155 tg mice have similar or lower levels of cytotoxic Perforin and Granzyme proteins compared to wt NK cells. Overexpression of miR-155 in primary mouse NK cells resulted in increased IFN-γ secretion after stimulation with IL-12 and IL-18. These data link miR-155 expression with stimuli that strongly induce IFN-γ in NK cells and suggest that

miR-155 functions as a strong positive regulator of IFN-γ production. Mechanistically, we observed downmodulation of SHIP1 that correlates with an increase in PI3K and ERK, which are signaling regulators of NK cell cytotoxicity. Collectively our data indicate that NK cells overexpressing miR-155 exhibit enhanced cytotoxicity which is likely due to an increase in activating signaling. Overall, we have shown significant differences in activation and homeostasis suggesting that miR-155 can play a major role as a positive regulator of NK cell effector function. The study shows that miR-155 plays an important role in activation of NK cell immune function with implications for treatment of cancer.

Introduction:

Chapter 1: The Natural Killer Cell

The human immune response has traditionally been classified into two categories: innate or adaptive immunity.[1] Innate immunity typically consists of cells originating from myeloid and lymphoid progenitors that function in a rapid, but non-specific manner. Adaptive immunity, on the other hand, consists of a specific, acquired response involving relatively long term memory that is carried out by two main players: T and B lymphocytes. Another major player in the human immune system, the natural killer (NK) cell is a large granular lymphocyte that is an important regulator of the body's response to malignancies such as cancer and infection.[2] NK cells are essential for the survival of the organism as evidenced by fatal infections resulting from NK-cell deficiencies in children.[3] Physiologically, they are considered to be important as the body's first line of defense against infection or malignant transformation and give the body time to mount a full scale adaptive immune response.[4] For this system of classification, the human natural killer (NK) cell has characteristics of both innate and adaptive immunity.[2,5] NK cells have deeply regulated functions with various levels of activation and inhibition mediated by receptor-ligand interactions and a large range of cytokines and chemokines.

There are two theories that govern NK cell regulation: "missing self" and "induced self"[6,7]. The "missing self" theory postulates that NK cells are able to recognize and targets cells that have reduced or improper expression of "self" molecules that are normally expressed on healthy native tissue.[8] These include major histocompatibility complex (MHC) and human leukocyte antigen (HLA) class 1 molecules. The "induced self" theory focuses on NK ligands that can be induced by cellular events such as stress[6,9]. In fact, there is evidence of both activating and

inhibitory receptors, which suggests that NK cell function is controlled by a complex milieu of activation and inhibition. NK receptors that are associated with activation include cluster of differentiation 16 (CD16), NKG2D and NKp46 while receptors associated with inhibition include members of the killer cell immunoglobulin-like receptor (KIR) and Ly49 families of receptors. Additionally, there is evidence of overlap between MHC class 1 expression and receptors associated with "induced self", which indicates that the two theories of NK regulation function cooperatively.[6] The complexity of NK cell physiology is not only seen in their regulation but also in their mechanisms of immune action.

Spontaneous Cytotoxicity and Associated Signaling:

The classical notion that NK cells are members of the innate immune system is largely based on the NK cell's ability to "spontaneously", rapidly, and directly lyse potentially harmful target cells without specific acquisition of immune activation. This mechanism is typically associated with NK receptors such as NKG2D and NKp46[6]. These receptors couple with a variety of tyrosine kinase based signaling events to result in NK cytotoxicity[10]. NKG2D, a well-studied natural cytotoxicity receptor, has been shown to noncovalently associate with DAP10 and additionally, in the case of mouse NK cells, immunoreceptor tyrosine-based activation motif (ITAM) bearing DAP12. Although DAP10 and DAP12 result in distinct signaling pathways, they often present redundancies in function based on some overlap in pathways, which characterizes a central theme to NK signaling[11]. In particular, activation of NK cell cytotoxicity can occur via both DAP10 and DAP12 dependent pathways, which ultimately converge on downstream activation of molecules such as Vav-1.[12-14] Predominantly, NKG2D binding to target cell ligand results in downstream phosphoinositide 3-kinase (PI3K) activation. PI3K is a

catalytic heterodimer implicated in mechanistic regulation of cell survival, activation, and cytotoxicity. PI3K triggers a set of cellular events associated with Erk phosphorylation and downstream lytic granule mobilization.[9,15,16] Of these lytic granules, Perforin first proceeds to permeabilize the target cell membrane to facilitate entry of lytic Granzyme proteins, which utilizes protease activity to facilitate target cell lysis.[17] In this way, NK cells are able to recognize and directly destroy cells that present ligands indicative of stress or malignant transformation such as viral infection or cancer.

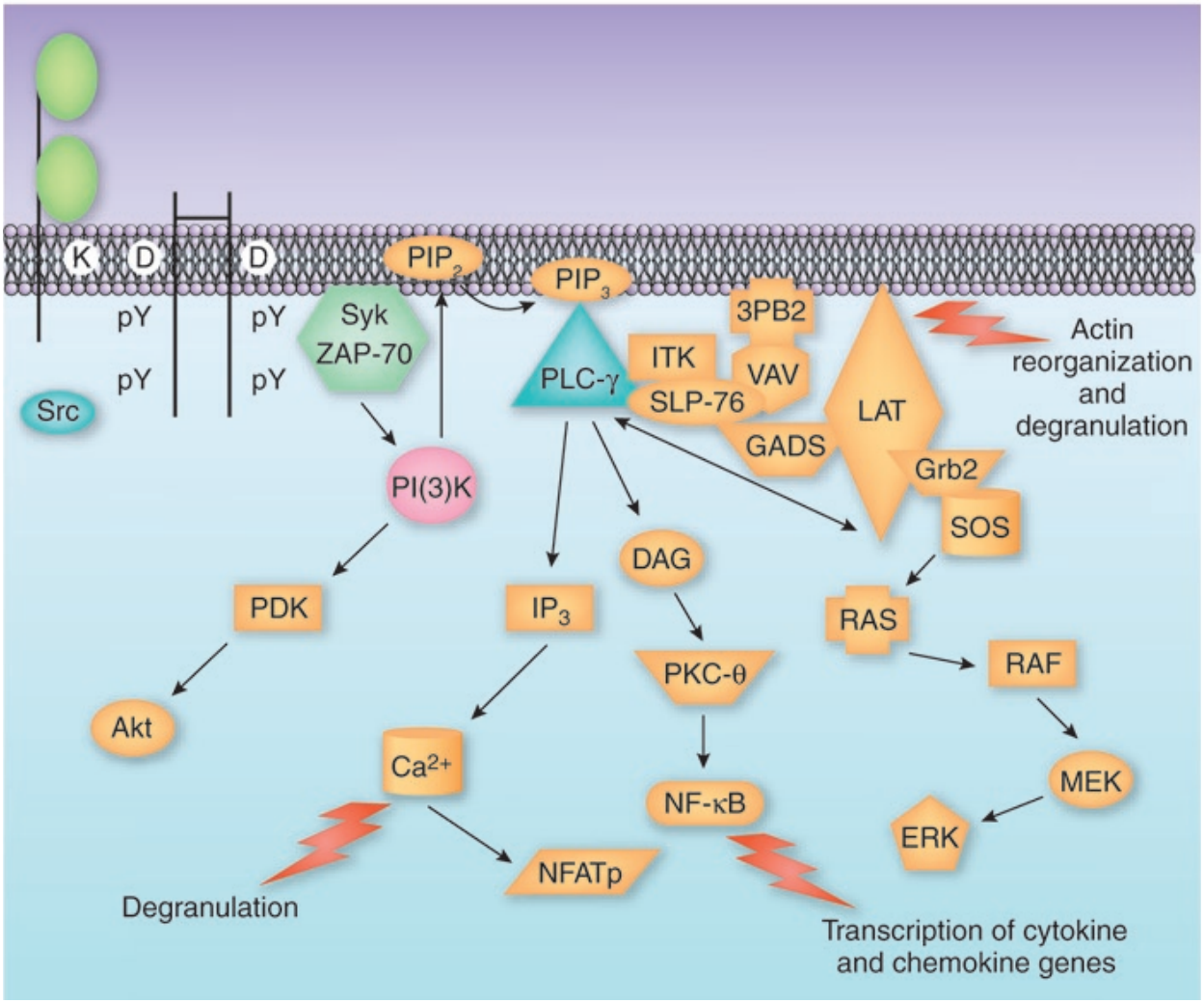

Figure i: Summary of ITAM based signaling in NK cells. Note PI(3)K and downstream Akt and ERK targets that are essential for NK cell effector functions.[9]

Antibody Dependent Cell Mediated Cytotoxicity:

The overall role of NK cells in the immune response goes far and beyond their "spontaneous" cytotoxic abilities. NK cells also express CD16, low-affinity Fcy receptor IIIA that binds to the Fc region of immunoglobulins such as IgG, which are usually bound to infectious agents, pathogens, or cancer cells. When CD16 forms an immunocomplex with IgG, it initiates a signaling cascade that ultimately results in NK cytotoxicity via molecules such as PI3K, Vav-1 and ERK in a similar but distinct manner to the previously mentioned spontaneous cytotoxicity[18]. This immunoglobulin-dependent form of cytotoxicity is known as antibody dependent cell mediated cytotoxicity (ADCC). In fact, NK cell ADCC is utilized in many current treatments for cancer. Currently, there are many examples of targeted antibody therapies for cancer including trastuzumab for breast cancers, rituximab for B cell lymphomas, bevacizumab and cetuximab for metastatic colorectal cancers, and ofatumumab in chronic lymphocytic leukemia.[19]

Cytokine Production:

NK cells are potent secretors of proinflammatory and immunosuppressive cytokines including interferon (IFN)-γ, tumor necrosis factor (TNF)-a and interleukin (IL)-10. In fact, IFN-γ , the classical NK cytokine, has been shown to be essential for T cell response and colocalization of dendritic cells (DC).[2] IFN-γ production is typically induced by a combination of monokines stimulatory signals and receptor-ligand activation.[2] Various monokines such as IL-1, IL-2, IL-12, IL-15, and IL-18 have been shown to be important regulators of IFN-γ production. In fact, its release can be initiated within minutes of monokines stimulation.[20] The release of IFN-γ by NK cells can in turn further activate the antigen presenting cells (APCs) such as monocytes, macrophages, and DCs to up-regulate MHC class I and increase APC cytokine secretion as

well.[1,2] The production of these monokines by monocytes, macrophages, and DC confirms the complexity of interactions between various effectors of an immune response.

Chapter 2: MicroRNA mediated regulation of NK cells

In the last few years, there is a large body of research indicating the possible role of microRNA (miR) in regulating immune response, with dysregulation at the root of disease.[2,21,22] First, RNA Polymerase II typically transcribes the primary transcript containing the miR sequence. This transcript is processed in the nucleus by the Drosha nuclease into a precursor miR and then exported into the cytoplasm. At this point, it is further processed by Dicer nuclease and then loaded into the RNA-induced silencing complex. This complex is able to target specific 3' untranslated regions (UTRs) of messenger RNA (mRNA) and repress translation or induce mRNA degradation.

MiR-155 and its target, SHIP1:

In humans, miR-155 is transcribed from the B cell integration cluster *MIR155HG* gene. It has been shown to play a complex role in regulation of immunity with evidence of both positive and negative effects associated with its modulation. A rapid induction of miR-155 is observed in B and T lymphocytes following antigen receptor activation suggesting that it is important for induction of immune response.[3,21] Further, it is required for the normal immune function of B, T, and dendritic cells as evidenced by severe immune deficiency upon its loss.[4,23] On the other hand, it is clear that the homeostatic balance of cellular miR-155 is crucial to proper function and signaling based on evidence that overexpression of miR-155 is linked to cases of B cell lymphoma, myeloid lymphoma, and myeloproliferative disorder in mice.[2,5,24,25] In mice given allogeneic hematopoietic stem cell transplants, miR-155 regulates the severity of consequent

acute graft versus host disease. This, along with other studies, suggests a proinflammatory role for miR-155.[6,7,26-29]

Recently there have been studies regarding the role of miR-155 in immune cells and particularly NK cells. In NK cells, miR-155 represses mRNA translation and/or induces mRNA degradation with one particular important regulatory molecule, SH2 domain containing inositol 5-phosphatase I (SHIP1).[8,30] SHIP1 is a negative regulator of activation and survival. While PI3K has been shown to convert the membrane phosphatidylinositol-(4,5)-bisphosphate (PI4,5P2) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) in order to initiate signaling associated with cell survival and activation, SHIP1 does the reverse and converts PI4,5P2 to PIP3 making it a negative regulator of NK cell activity.[6,9,31,32] Specifically in human NK cells, miR-155 is the most highly induced miR following costimulation with monokines, IL-12 and IL-18[6,30]. Interestingly, miR-155 is the only known microRNA that targets the 3'UTR region of SHIP1 mRNA.

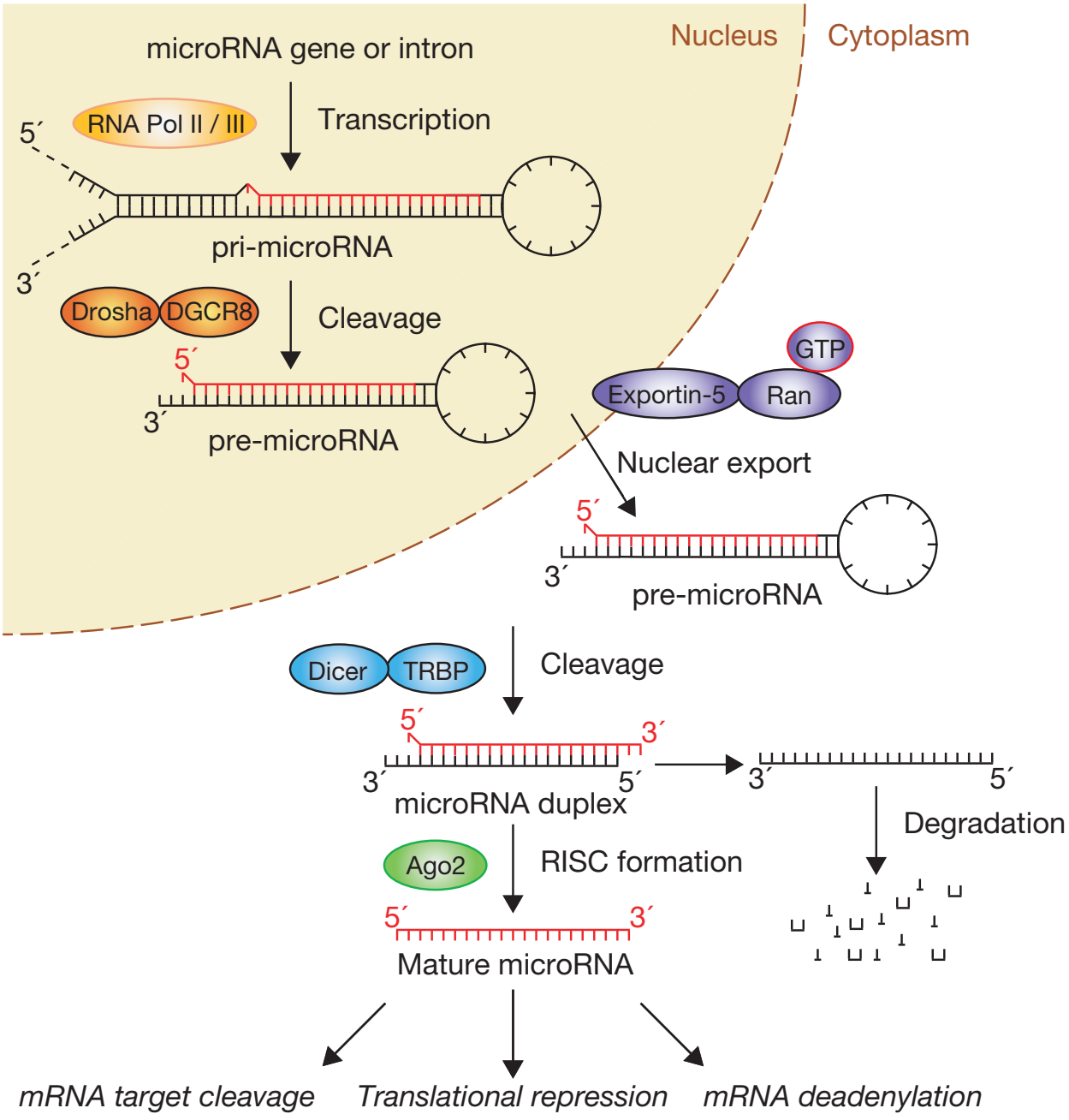

Figure ii: Pathway of microRNA processing [33]

Chapter 3: Approach

To recap, evidence suggests the miR-155 is essential for the human immune response and is associated with immune activation. Additionally, SHIP1, a known negative regulator of NK cell activation and cytotoxicity, is a primary target of miR-155. Thus, we hypothesized that miR-155 can act as a positive regulator of NK cell activity and cytotoxicity. In order to test this hypothesis, we utilized a mouse model overexpressing a miR-155 transgene off of the lymphocyte specific protein kinase (*lck)* promoter, which allows expression in specifically NK cell and T lymphocytes.

Materials and Methods:

Mice:

The *lck-miR-155* C57BL/6 (B6) tg mouse was used from a single founder as previously described.[6,26] Littermates 8.5-14 weeks were used for experiments. Animal work was approved by The Ohio State University Animal Care and Use Committee.

NK cell preparations:

All experiments were performed with purified primary mouse NK cells from splenic homogenate via microbead selection columns (Miltenyi Biotech Inc.) followed by FACS for NK1.1+ CD3ε- cell subset giving >99.5% purity using abs (clones) from BD Biosciences reactive against: NK1.1 (PK136) and CD3ε (145-2C11). Isolated mouse NK cells were cultured in RPMI-1640 (Invitrogen) containing 900 IU/ml of human IL-2 (Hoffman-LaRoche Inc.) and 55μM β-mercapethanol (Gibco) at 37 C. Splenic NK cell percentages and absolute numbers were determined by flow cytometric analysis of total splenic cell isolate for NK1.1+ CD3ε- NK cells. Analysis was performed with FlowJo v7.6.1 (TreeStar).

Cell lines:

The YAC-1 murine T-lymphoma cell line was maintained in RPMI-1640 (Invitrogen), and the murine mastocytoma P815 cells were maintained in DMEM medium (Invitrogen), both with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 2 mM L-glutamine (Gibco).

Cell stimulation.

Wt and miR-155 tg NK cells were stimulated with human IL-2 (90 ng/ml) or IL-12 (20 ng/ml; Genetics Institute Inc.) plus IL-18 (10 ng/ml; R&D Systems) for indicated times. For stimulation with YAC-1 tumor cells, NK cells were expanded in IL-2 for eight days, starved from IL-2 for 2 hours on ice to bring cells back to resting conditions, and mixed YAC-1 cells fixed with 1% paraformaldehyde at a 5:1 effector:target ratio and stimulated for the indicated times.

NK cell IFN-γ production:

Sorted subsets from wt and miR-155 tg NK1.1+CD3- NK cells were untreated or stimulated with indicated monokines Results are shown as the means of triplicate wells ± SEM.[34]

Cytotoxicity assays:

YAC-1 or P815 cells coated with an anti-mouse lymphocyte rabbit Ab (Accurate Chemical) were used as targets in a 4-hour ^{51}Cr release assay[10,35] with fresh NK cells or NK cells cultured in IL-2 90 ng/ml for 8 days.

Conjugate formation:

Immune complex formation among NK cells and YAC-1 tumor cells were examined by flow cytometry. YAC-1 tumor cells were infected using the GFP encoding lentivirus vector pCDH (System Biosciences) and sorted for GFP+ cells. For the conjugate assay, $2x10^5$ PE-labeled NK1.1+CD3- NK cells were mixed with $2x10^5$ GFP+ YAC-1 cells in 200 µl of cold RPMI-1640 with 10% FBS and centrifuged at 600 rpm for 1 min. To induce the formation of immune-complexes, cells were incubated at 37 C for 0 and 10 min. Reactions were stopped with ice cold PBS. NK1.1+ GFP+ conjugates were detected by FACS analysis.[11,36]

Western blot analysis:

NK cells were harvested and lysed into RIPA buffer or directly lysed in Laemmli buffer ($2x10^5$ cells/20 µl) as described22. Western blots were performed according to published protocols and Ab-reactive proteins were detected as described20. Abs used: the anti-SHIP1, anti-actin, and anti-Granzyme M (Santa Cruz Biotechnology); anti-Granzyme B, anti-perforin, anti-phospho-AKT^{Ser473}, and anti-phospho-$ERK^{Thr202/Tyr204}$ (Cell Signaling Technology).

Real-time RT-PCR:

RNA was extracted using Trizol (Invitrogen). Reverse transcription was performed with Taqman MicroRNA Reverse Transcription Kit and RT primers specific for miR-155 and U6 or 292 as control (Applied Biosystems). Real-time RT-PCR reactions were performed as described15. Data were analyzed with the comparative CT method using internal control U6 or 292 RNA levels to normalize differences in sample loading. Results (mean ± SEM of triplicate reaction wells) represent the n-fold difference of transcript levels in a particular sample compared to samples of wt NK cells.

Statistics:

Data were compared using Student's 2-tailed t-test. A p value < 0.05 was significant. Survival data were analyzed using Kaplan-Meier and long-rank test methods (GraphPad Prism Version 5.0).

Results:

Effect of miR-155 overexpression on NK cell number

To investigate the effects of miR-155 overexpression in NK cells, we used *lck*-miR-155 transgenic (tg) mice, which were previously generated.[12-14,26] We quantified miR-155 expression from RNA using RT-Realtime PCR obtained from NK1.1+ CD3- fluorescence activated cell sorting (FACS) for wt and miR-155 tg NK cells. We confirmed significantly higher values of miR-155 in the miR-155 tg NK cells compared to the wt NK cells (average induction of 26.6 +/- 5.3 fold; Figure 1A; $p<0.0001$, n=6). MiR-155 tg mice also had a higher percentage of splenic NK1.1+ CD3- NK cells compared to wt mice (Figure 1B, $p<0.0001$; n=16), and a higher absolute number of NK cells (Figure 1C; $p<0.0001$; n=13).

MiR-155 overexpression enhances NK cell cytolytic function:

We compared the ability of FACS sorted NK1.1+ CD3- NK cells from miR-155 tg and wildtype (wt) mice to kill the YAC-1 T cell lymphoma cell line and the RMA cell line expressing NKG2D ligand, Rae1B. Freshly isolated miR-155 tg NK cells lysed YAC-1 lymphoma targets ($p<0.01$, n=9) and RMA-Rae1B ($p<0.02$, n=5) with significantly higher efficiency compared to wt NK cells (Figure 2A). Additionally, miR-155 tg NK cells had significantly higher ADCC response against anti-lymphocyte antibody coated P815 mastocytoma cell line targets when compared to wt NK cells incubated with IL-2 for eight days (Figure 2B; $p<0.001$, n=5). To investigate potential cellular mechanisms responsible for the significantly enhanced NK cell cytolysis in miR-155 tg NK cells, we first analyzed protein levels of Granzyme B, Granzyme M and perforin by Western blot analysis. Interestingly, Granzyme B protein levels were significantly decreased in freshly isolated resting miR-155 tg NK cells compared to wt NK cells yet this downregulation

disappeared following activation in the presence of IL-2 for eight days (Figure 3C). It is worth noting that Granzyme B is not a known target of miR-155. No significant differences were otherwise observed in levels of Granzyme M and perforin in miR-155 tg vs wt NK cells. Effector-target conjugate formation is critical for NK cell cytotoxicity. As shown in Figure 3D, miR-155 tg NK cells have a significantly enhanced ability to form conjugates with YAC-1 cells when compared to wt NK cells (Figure 3D; $p<0.01$, n=3). This provides mechanistic evidence to partially explain the enhanced ability of miR-155 NK cells to kill tumor cell targets compared to wt NK cells.

MiR-155 in the regulation of IFN-γ production by IL-12 and IL-18 costimulated NK cells:
Indeed, miR-155 tg NK cells secreted greater amounts of IFN-γ when compared to wt NK cells following *in vitro* stimulation with IL-12 plus IL-18 monokines (Figure 4; $p<0.001$; n=6)

Effect of miR-155 on SHIP1 expression and PI3K associated signaling:
SHIP1 is downregulated in miR-155 tg mouse NK cells (Figure 5A). As discussed in Chapter 2 of the Introduction, SHIP1 is a negative regulator of the PI3K. AKT is a downstream target of PI3K and provides a method of measuring PI3K activity.[9,15,16] Following activation by either IL-2 or IL-15 (and occasionally in medium alone), AKT was visibly more activated by phosphorylation in miR-155 tg NK cells compared to wt NK cells (Figure 5B). ERK is also a downstream target of PI3K in NK cells and is associated with NK cell lytic function.[15,17] We found that its activation by phosphorylation was visibly higher in miR-155 tg NK cells compared to wt NK cells cultured in IL-2 or IL-15, and occasionally at rest in medium alone (Figure 5B). Since ERK signaling regulates cytotoxicity in NK cells we also quantified ERK phosphorylation

after binding YAC-1 tumor target cells. MiR-155 tg NK cells have higher levels of active, phosphorylated ERK compared to wt NK cells after conjugation with YAC-1 tumor target cells (Figure 5C). When stimulated with monokines or tumor target binding, miR-155 tg NK cells show enhanced activation of AKT and ERK when compared to wt NK cells.

Discussion:

NK cells are amongst the first immune cells to respond to a site of viral infection or malignant transformation. This rapid response, in conjunction with the NK cell's ability to recognize malignancies via MHC class 1 mismatch or induced NK receptor activation, give the NK cell a key role in immune surveillance against cancer. For these reasons, enhancement of NK cell activity presents a viable option for effective cancer therapies.

MicroRNA regulation is on the forefront of modulation of gene expression and control of intracellular signaling mechanisms. In this report, we investigated the role of miR-155 on the homeostasis and function of NK cells in an *lck*-miR-155 tg mouse model. Increased splenic percentage of NK cells and an overall increase in absolute number of splenic NK cells suggest evidence of a cellular homeostatic change. Presumably this is occurring due to miR-155 repression of SHIP1, a known negative regulator of cell survival, proliferation, and activation.[18,37] Although we later present data suggesting increased levels of NK cell cytotoxicity, this increase in NK cell number and percentage already presents an advantage to immune surveillance of cancer.

The observed increase in NK cell cytolytic activity against both YAC-1 and RMA-Rae1B T cell lymphoma lines can be due to various changes in NK cell phenotype and activation. Theoretically, miR-155 could be modulating expression of activating or inhibitory receptors on the NK cell surface or could be granting the NK cells a general heightened state of activation and metabolism. The evidence presented in this report suggests that miR-155 overexpression results in a heightened state of activation mediated through ERK signaling. Mechanistically, ERK has been associated with mobilization of lytic granules in the direction of the target cell to result in efficient lysis.[15,19] This brings up the issue of granule expression in the miR-155 tg NK cells

compared to those of the wt. Prior to activation during resting conditions, miR-155 tg NK cells express lower levels of Granzyme B protein compared to wt NK cells. The enhanced killing by freshly harvested miR-155 NK cells must not be affected by Granzyme B protein expression, which begs to question, what is causing the increased levels of cytotoxicity? It is seen that NK cells from Granzyme B-/- mice still express some cytotoxicity[2,38], suggesting that it is not solely essential for response. It is possible that other Granzymes are compensating for the loss of Granzyme B or that a rapid translational induction occurs after target cell binding. It is likely that miR-155 plays a large role in events leading up to lysis such as granule mobilization, cytoskeleton reorganization, and our data concerning increased levels of target cell conjugation with miR-155 tg NK cells. It is worth noting that the low expression of Granzyme B is most likely not due to direct repression and binding by miR-155 as the 3'UTR of Granzyme B mRNA does not have sufficient complementarity to allow for miR-155 binding. It has been shown that miR-223 directly binds to the 3'UTR of Granzyme B mRNA and miR-223 expression is downregulated by IL-15 activation.[2,39] The role that miR-155 plays in modulating NK cell homeostasis maybe be linked through the IL-15 receptor pathways, thus modulating miR-223 expression and Granzyme B protein levels. The interaction between Granzyme B and miR-155 needs further investigation.

The observation that miR-155 causes enhancement of not only spontaneous cytotoxicity, but also IFN-γ secretion and ADCC, suggests that its role in NK cell activation is broad and powerful. This is not surprising considering that miR-155 expression has been shown to be essential for normal immune function of B, T and dendritic cells.[20,23] Although these immune cells play different roles in regulating response to malignancy and pathogenic infection, there is some conservation of signaling which may be associated with the role of miR-155. Looking

specifically at NK cells, miR-155 negatively regulates SHIP1 and activates PI3K signaling, a known modulator of many NK cellular events associated with activation.[15] This activation can explain enhanced ADCC[40], increased IFN-γ secretion[31], as well as homeostatic modulation[41]. Looking specifically at IFN-γ secretion, our lab has specifically studied the role of SHIP1 in this key pathway. Utilizing NK92 human cell lines, we have observed that overexpression of SHIP1 via a Pinco vector decreases NK92 IFN-γ secretion compared to vector alone. This confirms our hypothesized roles of miR-155 and SHIP1 and confirms that an interaction between the two may be indeed the reason for our observed effects.

When comparing effects seen between spontaneous cytotoxicity and ADCC, we observe that different levels of activation are needed for both responses. This suggests that both processes, although similar in cytolytic effect, operate under unique methods of control. For spontaneous cytotoxicity, the rapid response and high levels of cytolytic activity witnessed in freshly isolated and resting miR-155 tg NK cells compared to wt aligns with the innate response of the NK cell and is the hallmark of its role in the first line of defense. It also suggests that miR-155, in this case, is playing a role in mediating the innate, direct, nonspecific response of the NK cell. However under activation with IL-2 for eight days, the differences between miR-155 tg NK cells and wt NK cells disappear. This is most likely due to long periods of activation at which point the NK cells may reach a maximum level of activity. Notably, high levels of activation do occur in the human body after the innate response has already started in the form of intercellular signaling via monokines and cytokines. Even in these instances of high activation where spontaneous cytotoxicity reaches a maximum, miR-155 manages to still modulate NK response via ADCC cytolytic pathways that more resemble adaptive immune pathways. Characteristic of adaptive pathways, NK ADCC is extremely low and difficult to detect at resting, freshly isolated

conditions. Still, we have observed higher levels of ADCC in the miR-155 tg mice compared to the wt. On the other hand, when the NK cell reaches a state of heightened activation via monokine stimulation, ADCC becomes more active, plays a larger role in cytolytic activity and is still higher in miR-155 tg NK cells compared to wt. Looking at how miR-155 is able to regulate both innate pathways that require low activation, and adaptive immune response that requires time and antibody presentation shows not only the versatility of the NK cell, but also the broad reach of microRNA regulation as witnessed with miR-155.

In addition to this, the full role of miR-155 in NK cells is still not well characterized. Many microRNA have hundreds of mRNA targets with complex cellular roles[42]. In T lymphocytes, miR-155 targets Suppressor of Cytokine Signalling 1 (SOCS1), which plays an important role in differentiation and activations[43]. If a similar event is occurring in NK cells, the downstream signaling would indeed be much more complex than simple PI3K activation.

In summary, we show the overexpression of miR-155 in NK cells positively regulates NK lytic function while modulating homeostatic balance. The elucidation of factors that control these processes is important for host defense. The selective enhancement of miR-155 in NK cells via direct delivery of miR-155 oligonucleotides via nanoparticle mediated control[44] could improve NK cell activation against progressive cancers or infections while avoiding the well-known toxicities that patients have encountered when undergoing systemic administration of NK cell-activating cytokines such as IL-2 or IL-12. Additionally, this could improve already existing antibody therapeutic for specific cancers that function via NK ADCC[19].

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Figure legends:

Figure 1. NK cell expansion in miR-155 tg mice. (A) $NK1.1^{+}CD3^{-}$ FACS sorted NK cells from spleen of wt and miR-155 tg mice were analyzed for miR-155 expression by Real-time RT-PCR. The graph is representative of 6 experiments with similar results. Results are expressed as the mean ± standard error mean (SEM) of triplicate wells. (B) Freshly isolated splenocytes of wt and miR-155 tg were stained with anti-NK1.1 and anti-CD3e Abs and analyzed by flow cytometry for percentage of $NK1.1^{+}CD3^{-}$ cells. (C) The absolute number of NK cells was calculated in spleens of wt versus miR-155 tg mice. The graph summarizes data from 4 wt and 2 miR-155 tg littermate mice. * Statistically significant; see text for details.

Figure 2. Effect of miR-155 overexpression on NK cell cytotoxic effector functions.

A) FACS sorted $NK1.1^{+}CD3^{-}$ NK cells from wt and miR-155 tg mice were used as effector cells in a 4-hour ^{51}Cr release assay using YAC-1 and RMA-Rae1b lymphoma target cells. E:T correlates with percent target cell lysis. B) Eight days after expansion in IL-2, sorted wt and miR-155 tg NK cells were tested for ADCC against ^{51}Cr labeled P815 mastocytoma IgG Ab coated targets cells. * Statistically significant; see text for details.

Figure 3. Effect of miR-155 overexpression on cytotoxicity associated NK functions

C) Resting (left) and IL-2 activated (8 days; right) $NK1.1^{+}CD3^{-}$ wt and miR-155 tg NK cells were analyzed for Granzyme B, Granzyme M and perforin protein levels by Western blot using actin as a loading control. D) Left: wt and miR-155 tg NK cells labeled with PE-conjugated anti-NK1.1 Ab were incubated with GFP^{+} YAC-1 tumor cells. Conjugate formation was analyzed 0 minutes and 10 minutes after incubation using flow cytometry. NK cell-target cell conjugates were gated and identified as $NK1.1^{+}GFP^{+}$ events. The percentages of conjugates are shown on top of the representative dot plots. Right: The graph summarizes the data of conjugate formation

obtained from three wt and three miR-155 tg NK cell samples co-incubated with YAC-1 tumor cells. * Statistically significant; see text for details.

Figure 4. The effect of miR-155 overexpression on NK cell IFN-γ production

Wt and miR-155 tg $NK1.1^{+}CD3^{-}$ NK cells were each co-stimulated in *vitro* for 18 hours at 37 C using IL-12 (20 ng/ml) and IL-18 (10 ng/ml) and supernatants were analyzed for IFN-g secretion by ELISA. * Statistically significant; see text for details.

Figure 5. Effect of miR-155 overexpression on SHIP1 protein expression and AKT and ERK activation in NK cells. A) $NK1.1^{+}CD3^{-}$ NK cells from wt and miR-155 tg mice were analyzed for SHIP1 protein levels by Western blot. Actin was assessed to ensure equal loading. B) $NK1.1^{+}CD3^{-}$ NK cells from wt and miR-155 tg mice were left untreated or stimulated with IL-2 (90 ng/ml) or IL-15 (100 ng/ml) for 10 minutes. Western blot analysis was performed on total lysates using anti-phospho-$ERK^{Thr202/Tyr204}$, anti-phospho-AKT^{Ser473} and actin Abs. C) $NK1.1^{+}CD3^{-}$ NK cells from wt and miR-155 tg mice and paraformaldehyde-treated YAC-1 cells were mixed and incubated at ratio 5:1 for the indicated times. Lysates from NK and YAC-1 samples were analyzed by Western blot using anti-phospho-$ERK^{Thr202/Tyr204}$ and actin Abs. These blots are representative of at least two independent experiments. * Statistically significant; see text for details.

Figure 1

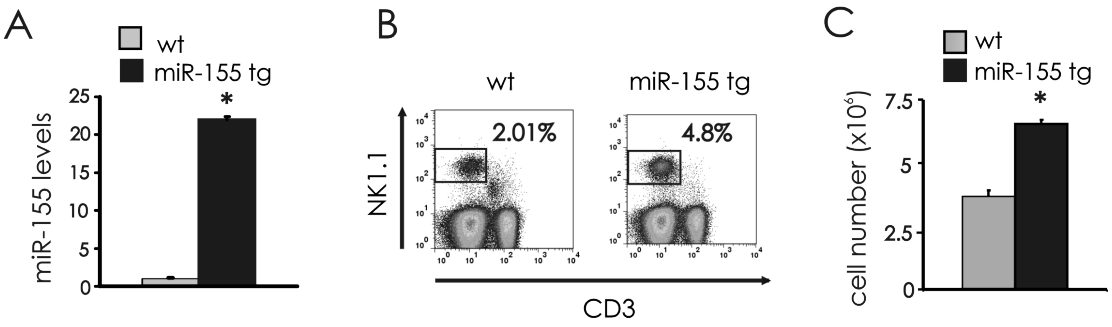


Figure 2

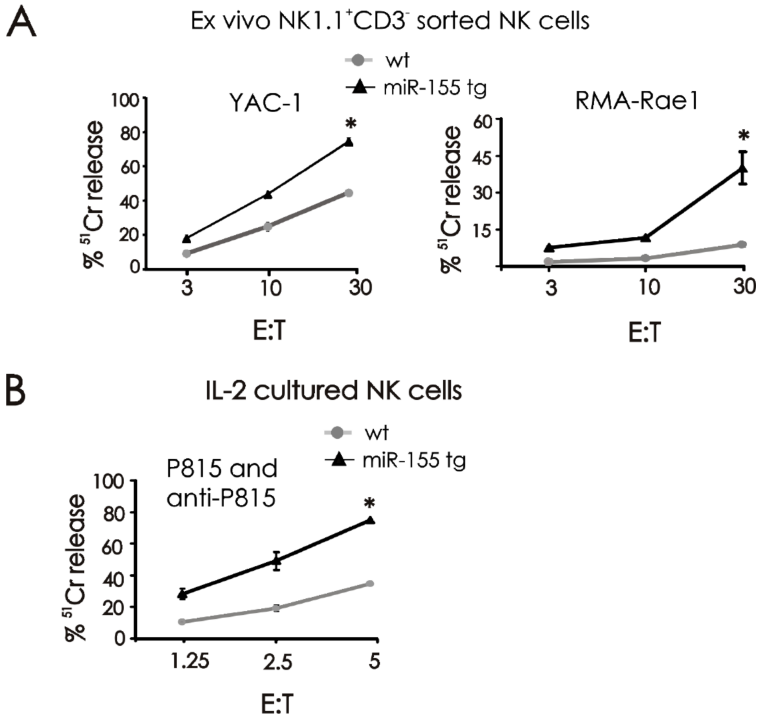


Figure 3

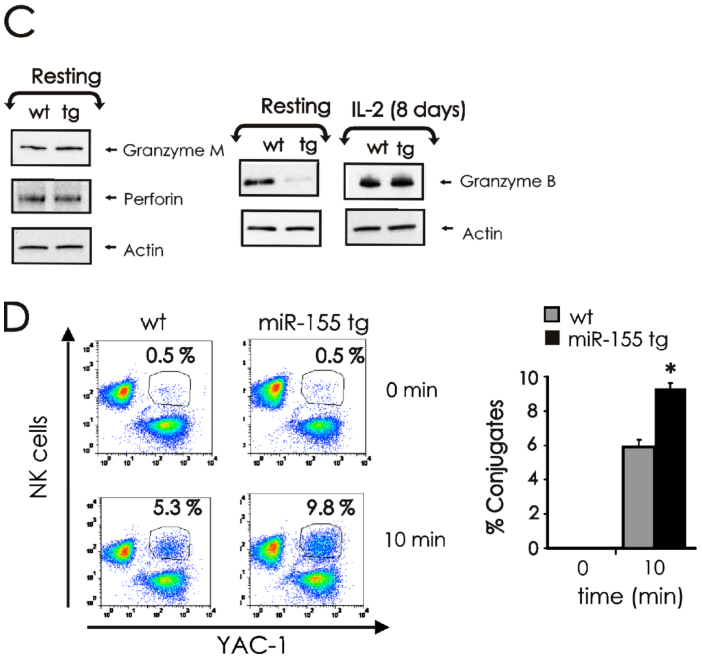


Figure 4

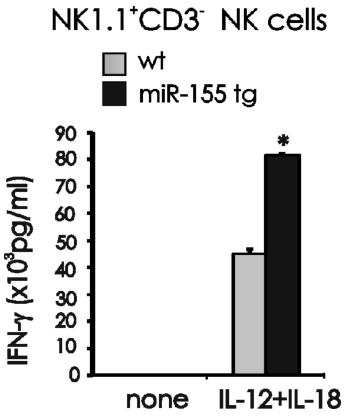
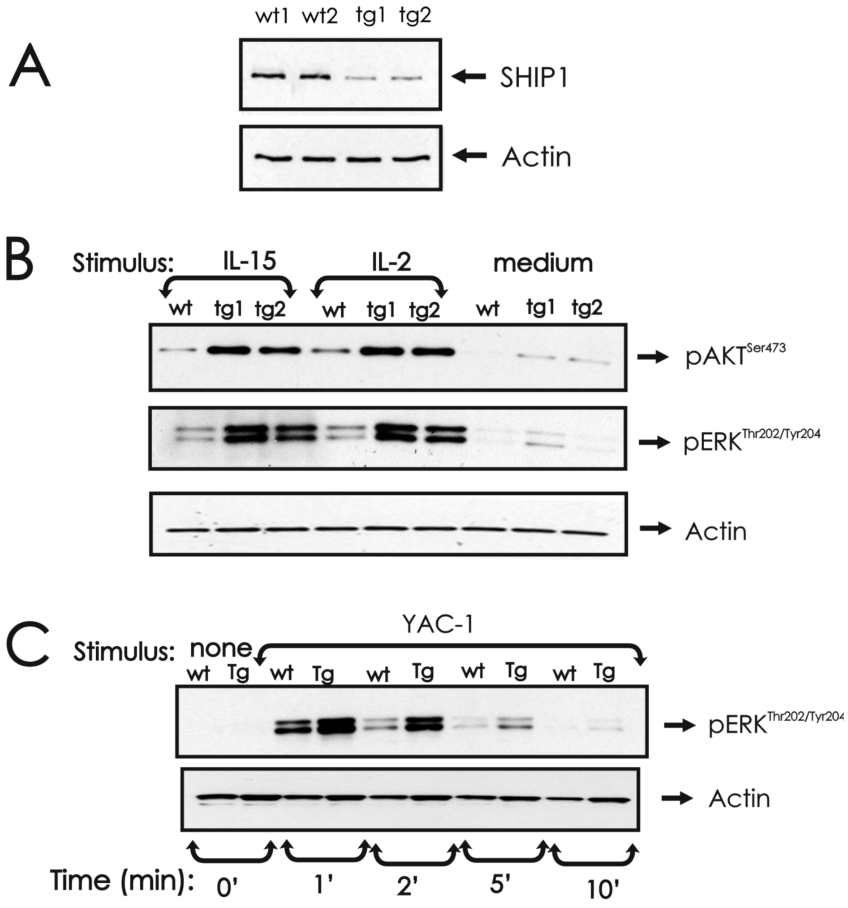


Figure 5


A
wt1 wt2 tg1 tg2
SHIP1
Actin
B
Stimulus: IL-15 IL-2 medium
wt tg1 tg2 wt tg1 tg2 wt tg1 tg2
pAKT Ser473
pERK Thr202/Tyr204
Actin
C
Stimulus: none YAC-1
wt Tg wt Tg wt Tg wt Tg wt Tg
pERK Thr202/Tyr204
Actin
Time (min): 0' 1' 2' 5' 10'